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Transformation of maize with the *p1* transcription factor directs production of silk maysin, a corn earworm resistance factor, in concordance with a hierarchy of floral organ pigmentation

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Summary

The maize *p1* gene encodes an R2R3-MYB transcription factor that controls the biosynthesis of red flavonoid pigments in floral tissues of the maize plant. Genetic and quantitative trait locus analyses have also associated the *p1* gene with the synthesis of maysin, a flavone glycoside from maize silks that confers natural resistance to corn earworm. Here, we show directly that the *p1* gene induces maysin accumulation in silk tissues. Transformation of maize plants that had low or no silk maysin with *p1* transgenes elevated silk maysin concentrations to levels sufficient for corn earworm abiosis. The *p1* transgenes also conferred red pigment to pericarp, cob, husk and tassel tissues, as expected; however, different subsets of these tissues were pigmented within individual transgenic plants. Statistical analysis shows that the pigmentation patterns observed amongst the *p1* transgenic plants conform to a hierarchy that is similar to the temporal ordering of floral organ initiation. We propose that the observed hierarchy of pigmentation patterns is conferred by variation due to epigenetic control of the *p1* transgenes. The production of plants with improved traits through genetic engineering can depend in large part on the achievement of tight organ-specific expression of the introduced transgenes. Our results demonstrate that the production of transgenic plants using a promoter with well-defined tissue specificity, such as the *p1* promoter, can result in unexpected variation in tissue specificity amongst the resulting transgenic plants.

Introduction

Corn, *Zea mays* L., is the most important cereal crop in the USA, and a major crop in world agriculture. For 2003, total global corn production was reported to be 593 952 thousand metric tons (http://www.corn.org/web/wcornprd.htm). Damage by insect pests can greatly decrease crop yield and grain quality. One of the major insect pests of corn is the corn earworm, *Helicoverpa zea* (Boddie). Corn earworm larvae initially feed on the exposed silks, but older larvae may burrow through the silk mass to the ear, where they consume the developing kernels. This damage predisposes the ear to fungal infestations, which can lead to serious food safety risks (Ortega *et al*., 1980). For example, aflatoxin, a mycotoxin produced by the fungus *Aspergillus flavus*, is carcinogenic to humans and animals (Dowd and White, 1995). To control corn earworms, growers may make 30 or more applications of insecticide per season (Cooke, 1997). As a result of the economic costs and environmental impact of widespread applications of synthetic pesticides, there has been considerable interest in the identification of maize germplasm with natural resistance to corn earworm. One type of natural resistance has been associated with the presence of maysin, a C-glycosyl flavone, in the silks (Waiss *et al*., 1979; Elliger

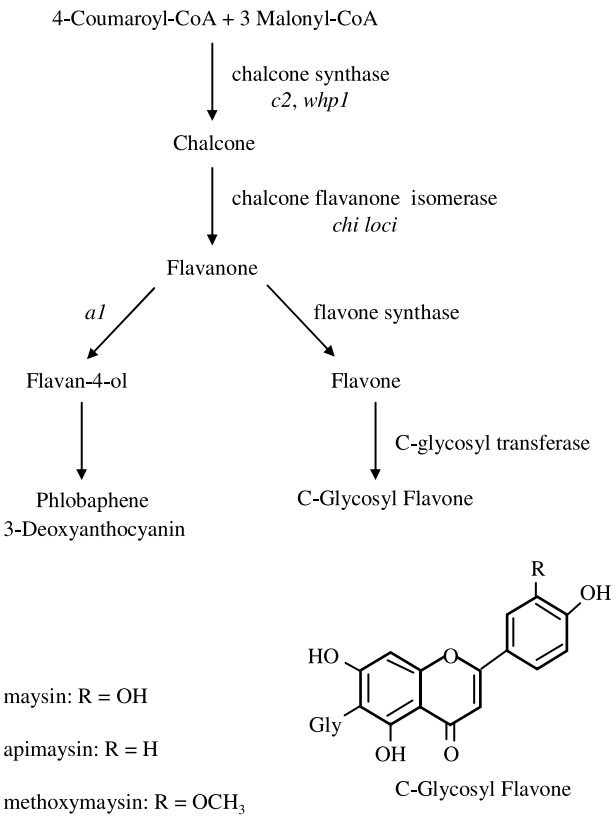

Figure 1 Proposed biosynthesis pathway for maysin and phlobaphenes. Modified from Lee *et al*. (1998) and Zhang *et al*. (2003).

et al., 1980; Snook *et al*., 1994) (Figure 1). Maysin is antibiotic to *H. zea* larvae and is thought to interfere with amino acid metabolism in the insect gut (Byrne *et al*., 1997).

Corn earworm resistance is positively correlated with a silk browning phenotype (Byrne *et al*., 1989, 1996a), in which the silks turn brown within minutes after wounding or cutting. This browning reaction is under monogenic control and is caused by the oxidation of phenolic compounds (Levings and Stuber, 1971). Previous genetic studies have indicated that the silk browning phenotype is related to the constitution of the *p1* locus (Coe *et al*., 1988). The *p1* gene encodes an R2R3-MYB domain transcription factor that regulates a branch of the flavonoid biosynthesis pathway (Figure 1), leading to red phlobaphene pigments in the cob and pericarp (Grotewold *et al*., 1991, 1994). Styles and Ceska (1989) showed that a functional *p1* allele specifies both red pigmentation and the production of C-glycosyl flavones, although the presence of maysin was not specifically determined. Further evidence that *p1* regulates maysin synthesis was obtained in a quantitative trait locus (QTL) analysis performed by Byrne *et al*. (1996b), which found that the *p1* locus coincides with a major QTL responsible for 58% of the phenotypic variance in maysin levels. In addition, experiments by Grotewold *et al*. (1998) showed that maize BMS (Black Mexican Sweet) callus cells transformed with a *p1*-expressing transgene accumulated C-glycosyl flavones related to maysin and 3-deoxy flavonoid precursors of phlobaphenes. More recently, expression of the maize *p1* gene and a tightly linked paralogous gene, termed *p2* (Zhang *et al*., 2000), in maize cell cultures induced the synthesis of phenylpropanoids and C-glycosyl flavones related to maysin (Zhang *et al*., 2003). However, neither study detected the production of maysin, leading the authors to suggest that the maize cell culture system used was not competent to express the functions required for glycosyl modifications of the flavonoid skeleton. Here, we provide direct evidence that the *p1* gene induces maysin production through analysis of transgenic maize plants. Maysin concentrations were elevated in maize plants transformed with *p1* transgenes relative to plants without a transgene. These results demonstrate the effectiveness of using a transcription factor to control an entire pathway for biosynthesis of an advantageous natural compound in transgenic plants.

Results

Transformation with a *p1* transgene induces maysin accumulation in maize silks

To directly determine whether *p1* regulates the production of maysin and maysin analogues in silks, the flavonoid contents of silks from a plant carrying a *p1* transgene were determined by reverse-phase high-performance liquid chromatography (HPLC) (Snook *et al*., 1989; Table 1). The transformation event analysed carried a transgene containing the promoter and

Table 1 Percentage of flavones in silks from a pWRWR transgenic plant*

Plant genotype	Chlorogenic acid	Iso-orientin/rhamnosyl	Maysin	Apimaysin	Methoxy-maysin
pWRWR†	0.088	0.006	0.637	0.008	0.009
Hill‡	0.063	0.000	0.000	0.000	0.000

*Values given in percentage fresh weight.

†Hemizygous T_1 plants that had been transformed and crossed with the Hill line.

‡Non-transformed Hill plant.

complementary DNA (cDNA) of the *P1-wr* allele. This transgene, designated pWRWR, conferred uniform dark red pigmentation in pericarp and cob, as well as uniform red pigmentation to dried husks, tassel glumes and silks (Cocciolone *et al*., 2000). The levels of maysin, apimaysin and methoxymaysin in silk from the transgene recipient line, HiII, were below the limits of detection. The presence of the pWRWR transgene induced detectable levels of the three compounds; however, the maysin concentration was greatly elevated (0.637% fresh weight) relative to apimaysin and methoxymaysin (0.008% and 0.009% fresh weight, respectively). Hence, the *p1* gene specifically controls maysin production in silk.

Maysin concentrations are associated with pericarp pigmentation in *p1* transgenic plants

Previously, we described the production and molecular characterization of plants transformed with various promoter/cDNA combinations of two different *p1* alleles: *P1-rr* and *P1-wr* (Cocciolone *et al*., 2001). The transgene constructs, pRRARR, pRRAWR, pWRARR and pWRAWR (Figure 2), are collectively referred to as P::P transgenes. Here, we present a detailed analysis of the transgene-conferred pigmentation patterns, and the relationship of pigmentation intensity and distribution to the concentration of maysin in silk.

The P::P transgenes conferred three spatial patterns of pericarp pigmentation: uniform, present throughout the pericarp (Figure 3B); white cap, predominantly in the gown or lower half of the kernel (Figure 3C); and silk scar, only at the silk attachment region (Figure 3D). These patterns occurred independently or in combination (Figure 3E). Pericarp colour also varied amongst individual transgenic plants, ranging from light orange to very dark red (Figure 3, top). Because maize silk tissues are derived by outgrowth of the two anterior carpels that give rise to the pericarp covering the germinal face of the kernel (Kiesselbach, 1949), we asked whether the pericarp pigmentation phenotype and silk maysin levels were correlated. To address this question, silk maysin levels, pericarp colour intensity and pigmentation pattern were scored for a subset of 41 T_1 plants from 16 independent transgenic lines generated with the four P::P constructs. Pericarp colour intensity was ranked on a scale devised by Brink and Styles (1966) and recreated here (Figure 3, top). The scale ranges from 1 to 11, with a score of 1 corresponding to the absence of pigment and a score of 11 corresponding to the darkest red colour. On this scale, the pericarp pigmentation of the P::P transgenic plants ranged in score from 3 to 11.

Silk maysin concentrations for the 41 T_1 plants are represented graphically as a percentage of the fresh silk weight in Figure 4. The value above each bar represents the pericarp colour score for a given plant, and the pigmentation patterns are listed below the graph. In all cases, the T_0 plants had been crossed with pollen from the inbred line 4Co63, which carries a *p1-wwb* allele that confers white pericarp, white cob and

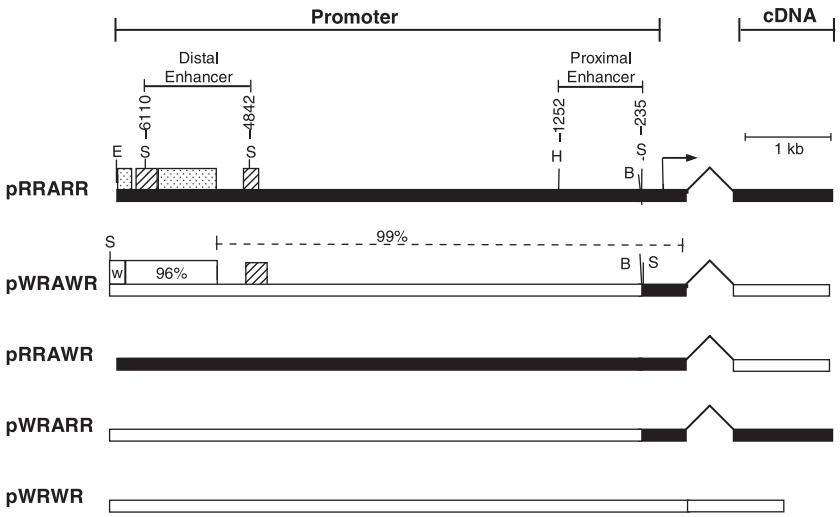

Figure 2 Diagram of the P::P transgene constructs. The promoter and complementary DNA (cDNA) regions of the P::P constructs are indicated by black boxes for *P1-rr* sequences and white boxes for *P1-wr* sequences. A comparison of the upstream regulatory regions of *P1-rr* and *P1-wr* is shown schematically for the first two constructs. The *P1-rr* upstream regulatory sequence contains two functionally defined enhancer regions: a 1.0 kb proximal enhancer (–235 to –1252) and a 1.2 kb distal enhancer (–4842 to –6110) (Sidorenko *et al*., 1999, 2000). The dotted boxes are sequences unique to *P1-rr*, and the box marked 'w' is specific to *P1-wr*. Hatched boxes indicate a homologous sequence in *P1-rr* and *P1-wr* that is partially duplicated in *P1-rr*. The open box region in *P1-wr* is 96% homologous to a segment of *P1-rr* that is located 1.1 kb upstream of the *Eco*RI site. The broken line delineates a region of 99% sequence homology. The bent arrow indicates the location of the transcription start site of *P1-rr*. The bent lines represent intron 1 of the maize *adh1* gene. Restriction enzymes are indicated as E (*Eco*RI), S (*Sal*I), H (*Hin*dIII) and B (*Bgl*II).

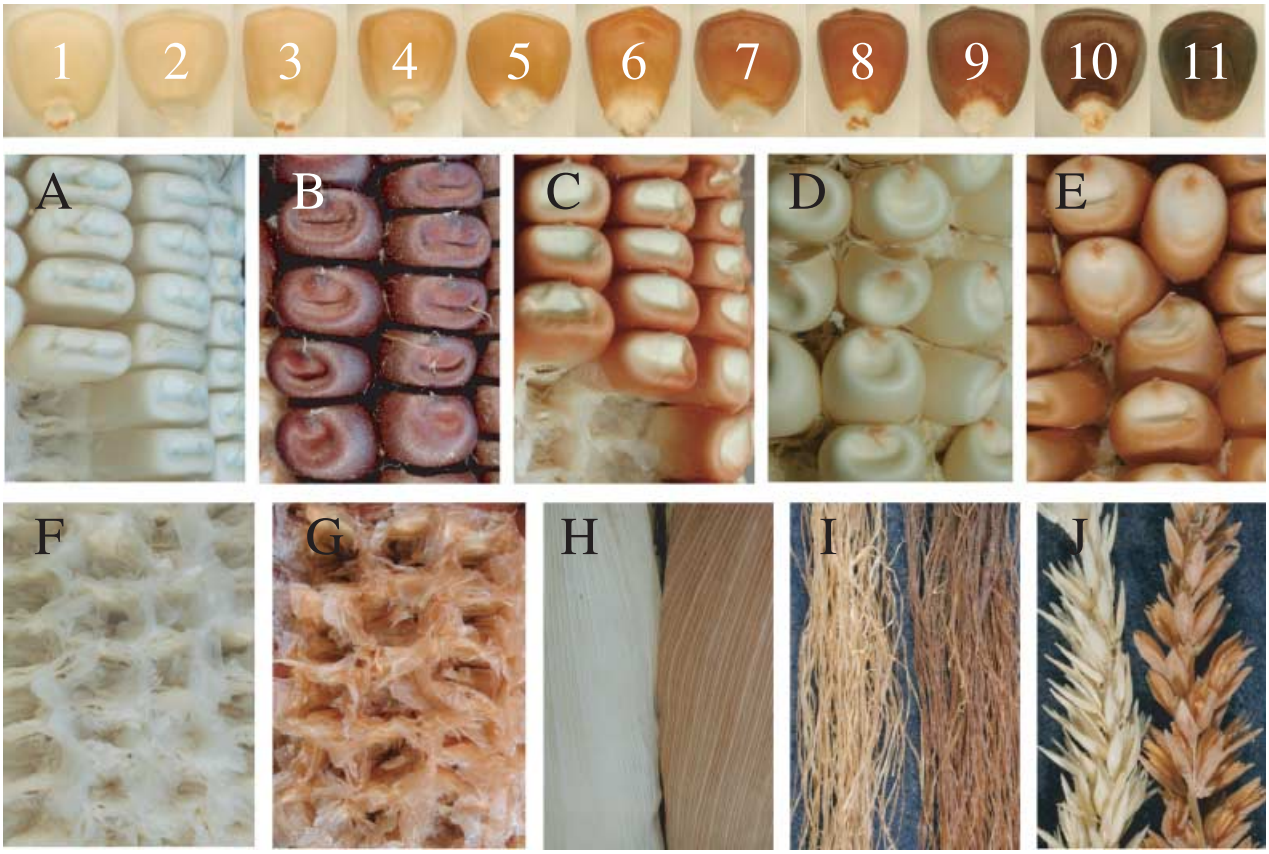

Figure 3 Maize kernel and plant phenotypes. Top: pericarp colour intensity scale showing the spectrum of pericarp pigmentation amongst natural *p1* alleles. Bottom: phenotypes of kernels produced on P::P transgenic plants. (A) Kernel phenotype of untransformed plant. (B–E) Pericarp pigmentation patterns conferred by P::P transgenes: (B) uniform red pericarp; (C) gown pigmentation; (D) silk scar pigmentation; (E) combination of silk scar and gown pigmentation. (F, G) Cob phenotypes: untransformed plant (F) and plant transformed with P::P transgene (G). (H–J) Phenotype of untransformed (left) vs. transformed (right) for husk (H), silks (I) and tassel (J).

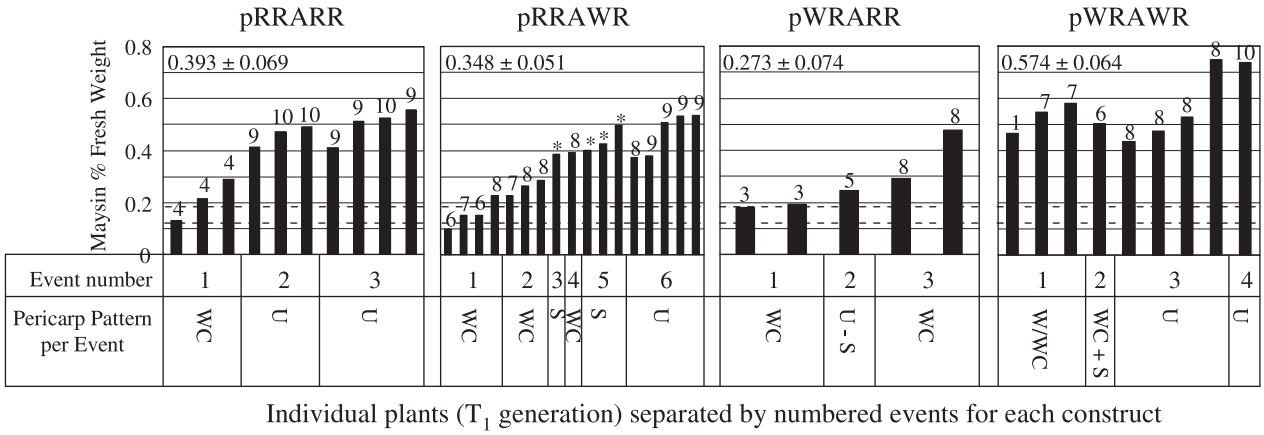

Figure 4 Comparison of pericarp pigmentation intensity and organ pattern with the concentration of silk maysin for P::P transgenic plants (T_1 generation). Each graph depicts data from one transforming plasmid, listed above the graph. Silk maysin concentration as a percentage of fresh weight is graphed for individual plants, which are grouped by numbered transformation events listed below the graph. Estimated mean maysin values for each construct are in the upper left corner of each graph. Broken lines indicate the range of maysin values obtained for control plants without transgenes. Numbers above the bars are the pericarp colour scores determined using the scale from Figure 3; an asterisk indicates that only the silk scar was coloured. Pigmentation patterns for each transformation event are given below the graphs: WC (white cap); U (uniform; including silk scar colour); U – S (uniform; excluding silk scar colour); S (silk scar only); W (white).

browning silks. Maysin concentrations were determined for three plants from the T_1 population that lacked the transgene due to segregation in order to determine the background silk maysin levels; these values ranged from 0.125% to 0.187% fresh weight.

Plants with darker pericarp colour (scores = 7) tended to have higher maysin values, whereas plants with lighter pericarp colour tended to have lower maysin values. The pericarp colour score was statistically significant (*t*-statistic = 4.51, d.f. ≈ 21.9, *P* value = 0.0002) when added to the model in Equation (1) ('Experimental procedures'). This indicates a significant positive correlation between maysin concentration and pericarp colour score when plasmid and event effects are accounted for. However, this correlation was not absolute. For example, one plant transformed with pRRAWR had a pericarp colour score of 7, but had < 0.2% silk maysin, whereas one plant transformed with pWRAWR had no pericarp colour (score = 1), but > 0.4% silk maysin. Plants with the following pigmentation patterns tended to consistently have high maysin values (ranging from 0.372% to 0.747% fresh weight): silk scar (S), uniform (U) and white cap with silk scar (WC + S). The one exception to this was a plant that had uniform pigmentation but no silk scar pigmentation (U – S). Plants with the white cap-only pattern had either high or low maysin values.

Transgenes confer hierarchical pigmentation amongst organs

The endogenous *P1-rr* allele confers pigmentation not only to the kernel pericarp and cob, but also to the husks, silks and tassel glumes of the mature dried plant. To determine which of these organs are pigmented by the P::P transgenes, these tissues, with the exception of silk, were scored for the presence of detectable pigmentation. The silk tissue could not be scored for transgene-conferred coloration because the T_1 population carried a *p1-wwb* allele, which promotes silk browning. Of the 75 T_1 plants that had a detectable phenotype, only 26 plants (35%) exhibited pigmentation in all four organs scored – pericarp, cob, husk and tassel glumes (Figure 3F–J). The remaining 49 plants (65%) had pigmentation in specific subsets of these organs, and in some cases the pigmentation patterns even differed amongst sibling plants. Such variability in transgene phenotype amongst floral organs has been reported previously for plants transformed with a *P1-rr* promoter driving a GUS reporter gene (P::GUS; Cocciolone *et al*., 2000). The spatial patterns conferred by the P::GUS transgene were classified into five groups that fitted into a hierarchical ordering, in which each successive group had GUS activity in one more organ than the previous group – starting with pericarp only, followed by the sequential addition of GUS activity in cob, husk, silk and tassel glumes.

The pigmentation patterns of the P::P transgenes were compared with the hierarchical GUS activity patterns of the P::GUS transgenes. All of the P::P transgenes, irrespective of the allelic origin of the promoter and cDNA, produced patterns consistent with the hierarchy observed for the P::GUS transgenes. Of 75 plants from 23 independent transformation events, 69 plants from 20 events had transgene-conferred pigmentation patterns consistent with the hierarchy (92%), and only six plants from four events had patterns inconsistent with the hierarchy (Table 2). The presence of hierarchical pigmentation patterns was not correlated with specific differences in transgene copy number, as determined by DNA gel blot analysis (data not shown).

Transgene-conferred hierarchy unlikely to be due to chance

A statistical test was developed to determine whether the apparent hierarchical ordering of the P::P transgene data could be explained by chance. The null hypothesis of our test was that pigmentation in the tassel, husk, cob and pericarp

Table 2 Analysis of the pigmentation patterns of the P::P transgenic plants*

	Hierarchical patterns				Non-hierarchical patterns					
Plasmid	P	PC	PCH	PCHT	Total† in hierarchy	CH	PCT	PT	Total deviant	Total†
pRRARR	4 (2)	0	5 (1)	4 (1)	13 (4)	0	0	0	0	13 (4)
pRRAWR	1 (1)	11 (2)	0	8 (2)	20 (5)	0	2 (2)	3 (1)	5 (3)	25 (8)
pWRARR	9 (4)	2 (1)	6 (3)	3 (2)	20 (7)	0	0	0	0	20 (7)
pWRAWR	3 (1)	0	2 (1)	11 (3)	16 (4)	1 (1)	0	0	1 (1)	17 (4)
Total	17 (8)	13 (3)	13 (5)	26 (8)	69 (20)	1 (1)	2 (2)	3 (1)	6 (4)	75 (23)

Data are presented as the number of plants with a given pigmentation pattern, followed in parentheses by the number of independent events represented.

*T_1 generation.

†Some events contain plants with different patterns, and so the total number of events is less than the sum across the pigmentation patterns.

was mutually independent. An alternative hypothesis was that the patterns conformed to the proposed hierarchy of PCHST (pericarp, cob glume, husk, silk and tassel; Cocciolone *et al*., 2000). For example, let us consider two groups of plants: those with pigmentation in the tassel and those without tassel pigmentation. Under the null hypothesis, plants from both groups are equally likely to have pigmentation in the husk. Under the alternative hypothesis, plants in the first group are more likely to have pigmentation in the husk than plants in the second group. We used the phenotypic data of the 75 transgenic plants from 23 independent lines that were scored for the presence or absence of pigmentation in the pericarp, cob, husk and tassel. To test our hypotheses, we conditioned on the observed number of plants having pigmentation in each tissue: 74 with red pericarp, 55 with red cob, 40 with red husk and 31 with red tassel. To determine the chance that 69 of 75 plants would follow the proposed expression hierarchy under the null hypothesis of independence, we carried out the following procedure 10 000 times to obtain an approximate answer.

1 Seventy-four of the 75 plants were randomly chosen to exhibit transgene-conferred pigment in the pericarp.

2 Fifty-five of the 75 plants were randomly (and independently of step 1) chosen to exhibit transgene-conferred pigment in the cob.

3 Forty of the 75 plants were randomly (and independently of steps 1 and 2) chosen to exhibit transgene-conferred pigment in the husk.

4 Thirty-one of the 75 plants were randomly (and independently of steps 1, 2 and 3) chosen to exhibit transgene-conferred pigment in the tassel.

5 The number of randomly generated plants that exhibited phenotypic patterns consistent with the proposed hierarchy was counted.

In 10 000 trials, the observed number of plants following the hierarchy was never as large as 69, the value of the actual data. The maximum number of plants following the hierarchy in the 10 000 trials was 65. The distribution of the 10 000 values had a normal shape, a mean of 49.3 and a standard deviation of 2.9. The test clearly indicates that the observed number of plants having phenotypic patterns that agree with the hierarchy would be highly unusual under the independence model. Thus, we conclude that the underlying model for the current data has significantly greater tendency to produce observations consistent with the hierarchy than does the independence model.

The analysis above ignores the relationship of plants from the same transgenic event. As the spatial patterns of plants originating from the same transformation event tended to be more similar than patterns for plants from different events, perceived evidence in favour of the hierarchy could be inflated by repeated observations of a pattern satisfying the hierarchy within a particular event. To address this possibility, we conducted the analysis using event-level data, in which all of the patterns observed within a single transformation event were represented by the predominant pattern. For 19 of the 23 events, all plants within an event exhibited identical phenotypic patterns. For the other four events, three events had plants with exactly two distinct patterns, both of which agreed with the hierarchy. In these cases, the most common pattern was selected to represent the event. The remaining event contained a single plant out of four plants that had a pattern that was inconsistent with the hierarchy. That pattern was selected to represent the event so that our analysis would be conservative. Of the 23 patterns associated with the 23 events, 19 satisfied the expected hierarchy. To judge the statistical significance of this number, we conducted 10 000 random trials as described above using the 23 event patterns as the basic units for analysis. In 13 of the 10 000 trials, the observed number of events following the hierarchy was as large as 19, whilst the maximum number of events following the hierarchy was 20. The distribution of the 10 000 values had a normal shape, a mean of 13.5 and a standard deviation of 1.7. The test clearly indicates that the observed number of events whose phenotypic pattern agrees with the hierarchy using event-level data would be highly unusual under the independence model (P value $\approx$ 0.00013), further supporting our previous conclusion.

Presence of silk maysin also fits hierarchy

If silk maysin production corresponds to the 'silk' position in the hierarchy (PCHST), which lies between husk and tassel, then plants with pigmentation in the pericarp, cob, husk and tassel (PCHT) should have high silk maysin levels, and plants with pigmentation limited to pericarp and cob tissues (P and PC) should have background maysin values. Plants with pigmentation in the pericarp, cob and husk, but not the tassel (PCH), may or may not express the transgene in silk, and so could have either high or low silk maysin.

To determine whether the maysin data of the 41 T_1 plants described above fit with this idea, the data were re-analysed. A dot plot of the maysin values for plants in each of the categories P, PC, PCH and PCHT shows that all of the plants that exhibited pigmentation in the pericarp, cob, husk and tassel (PCHT) also had high silk maysin concentrations, ranging from 0.372% to 0.747% fresh weight (Figure 5). Plants with pigmentation in the pericarp only, or in the pericarp and cob

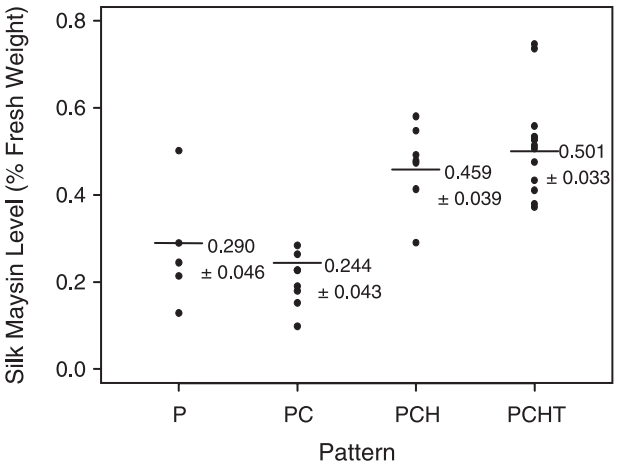

Figure 5 Dot plot of maysin concentration grouped by hierarchical pattern. Each point represents a single plant. Numerical values are the estimated mean maysin level for each pattern category. Tissues are given as P (pericarp), C (cob), H (husk) and T (tassel).

(P or PC), tended to have low maysin concentrations, with all but one value below 0.290% fresh weight. In the latter group, the endogenous *p1-wwb* allele from the 4Co63 genotype probably confers the observed maysin production (as described previously). As the T_1 plants are not in an isogenic background, some variation in maysin levels is expected due to segregation of other loci affecting maysin synthesis (Snook *et al*., 1993); however, there is almost no overlap in maysin concentrations between the low maysin (P, PC) vs. high maysin (PCHT) classes.

To determine the statistical significance of these observations, we added a 'pigmentation pattern' factor with four categories (P, PC, PCH and PCHT) to the mixed linear model described in Equation (1) ('Experimental procedures'). Estimated means were calculated for each of the pigmentation pattern categories (Figure 4). Contrasts amongst the means revealed the following facts. There was no significant difference between the means for patterns P and PC (t = 0.59, d.f. ≈ 6.1, P value = 0.5734) and the means for patterns PCH and PCHT (t = 0.72, d.f. ≈ 5.1, P value = 0.5027). The mean for pattern PCH was significantly greater than the means for patterns P and PC (t = 3.39, d.f. ≈ 5.8, P value = 0.0157). Likewise, the mean for pattern PCHT was significantly greater than the means for patterns P and PC (t = 4.68, d.f. ≈ 4.5, P value = 0.0072). All of these results are consistent with the idea that silk maysin synthesis conforms to the proposed organ-specific hierarchy of expression of the P::P transgenes.

Discussion

The maize *p1* gene encodes an R2R3-MYB homologous transcriptional activator of genes required for flavonoid biosynthesis. Mutations in *p1* result in a loss of phlobaphene, a flavonoid-derived pigment commonly found in maize floral organs, whilst mutations in *p1* and a tightly linked paralogous gene (*p2*) result in complete loss of maysin and related C-glycosyl flavones (Zhang *et al*., 2003). Previous studies have shown that a major QTL determining the levels of silk maysin coincides with the maize *p1* locus (Byrne *et al*., 1996a,b, 1997, 1998; Guo *et al*., 2001). Moreover, three independent studies have shown that expression of the *p1* gene in maize cell cultures activates a subset of genes required for flavonoid biosynthesis and results in the production of significant levels of flavones (Grotewold *et al*., 1998; Bruce *et al*., 2000; Zhang *et al*., 2003). It was surprising, therefore, that in none of the three previous studies did *p1* expression induce the formation of phlobaphene pigments or maysin in maize cell cultures. The lack of production of maysin was suggested to be due to an absence of cell-type specific factors required for glycosylation of the flavonoid skeleton (Grotewold *et al*., 1998). We show here that plants carrying *p1* transgenes induce the production of phlobaphene pigments in kernel pericarp and cob, and the accumulation of maysin in silks. These results provide a direct confirmation of the role of the *p1* gene in maysin production.

It is intriguing why maysin is not produced at detectable levels in the *p1*-transformed maize cell cultures. Possibly, maize cultured cells may not be physiologically or developmentally competent to express the flavonoid glycosylating activities. Alternatively, if maysin and/or its related glycosylated flavones are cytotoxic, then only those transformed cells that fail to induce these activities will survive for analysis. In either case, our findings indicate that cell culture systems are, to some extent, limited in their capacity to produce certain secondary metabolites.

Silk maysin levels greater than 0.2% fresh weight are sufficient for corn earworm antibiosis and reduction of corn earworm larval weights to < 50%, whilst levels greater than 0.4% fresh weight reduce larval weights to < 70% of controls (Wiseman *et al*., 1992). Transformation of maize with *p1* transgenes resulted in the elevation of silk maysin levels, relative to the no transgene controls, with values ranging from 0.372% to 0.747% fresh weight. Thus, the silk maysin concentration of the *p1* transgenes would be expected to confer resistance to corn earworm. However, a possible problem with using *p1* transgenes for corn earworm management is the associated pigmentation trait. Use of a silk-specific promoter would limit the production of maysin and associated pigments to the maize silk, and thus result in a product that may be useful in the sweet corn industry. Although high-maysin corn lines have recently been made available for

commercial germplasm improvement (Widstrom and Snook, 2001), a potential advantage of a transgenic approach is that the high-maysin trait conferred by the *p1* transgene may be introduced directly into elite lines pending improvements in transformation technologies. It is also crucial that any antibiotic trait in the silks be combined with husk and ear traits that help confer resistance; loose husks allow corn earworms to circumvent the silks and feed directly on the ear (Rector *et al*., 2002).

Transformation with constructs containing the upstream regulatory region of either *P1-rr* or *P1-wr* resulted in a hierarchy of transgene-conferred expression patterns amongst five floral organs – pericarp, cob, husk, silk and tassel – irrespective of whether the *p1* coding sequence (this study) or the GUS reporter gene (Cocciolone *et al*., 2000) was used. The hierarchical ordering is nearly the reverse of the timing of floral organ initiation, suggesting that there is an underlying developmental mechanism controlling *p1* promoter expression in transgenic plants (Cocciolone *et al*., 2000). Such developmental programming occurred independently of the genomic position of the transgene, because the majority of independent P::P transgenic lines (20 of 23) produced plants (69 of 75) with pigmentation patterns that fitted the hierarchical ordering. However, transgene structure and/or the genomic context probably influenced which pattern in the hierarchy was expressed, because plants derived from the same transformation event tended to have similar pigmentation patterns. Variation in the patterns amongst plants from the same transgenic event was observed, but it occurred more frequently for the P::GUS transgenes (Cocciolone *et al*., 2000). The lack of observed variegation within organs indicates that the transgene expression state for a particular organ is determined prior to organ formation. Hence, although the developmental programme can be shifted amongst patterns within the hierarchy, the pattern is set very early in development so that all cells follow the predetermined plan. The presence of hierarchical patterning with the use of different coding sequences, as in the case of P::P and P::GUS transgenes, suggests that the *P1-RR* and *P1-WR* promoters are susceptible to similar epigenetic regulation when placed in an artificial transgenic environment. The tissue-specific regulation of the natural *p1* alleles, *P1-wr* (Chopra *et al*., 1998; Cocciolone *et al*., 2001) and *P1-pr* (Das and Messing, 1994; Lund *et al*., 1995), has been attributed to epigenetic regulation; however, they do not conform to the hierarchical pattern reported here.

Maysin production fitted the 'silk' position of the hierarchy, in which plants with pericarp (P) and pericarp and cob (PC) patterns generally had low maysin levels, plants with a pericarp, cob and husk (PCH) pattern had either low or high maysin levels, and plants with a pericarp, cob, husk and tassel (PCHT) pattern all had high maysin levels. There was almost no overlap in maysin concentrations between the low maysin (P, PC) vs. high maysin (PCHT) classes. The one exception to this was a plant that had pericarp-only pigmentation but a high maysin level (0.502% fresh weight). The pericarp phenotype of this plant was both white cap and silk scar, with a pericarp colour score of 5. Such a low colour score was generally associated with low maysin values; however, the presence of a silk scar was associated with high maysin values. Because silk is derived as an outgrowth of the anterior carpels which also form the pericarp, the presence of pigment at the point of attachment of silk to kernel (silk scar) could be an indicator of *p1* expression in the silk. A prediction would be that plants with silk scar pigmentation would have high maysin levels irrespective of the pattern of pigmentation in other tissues. This idea is supported by the observation that four plants that exhibited pigmentation only in silk scar, and thus did not conform to the hierarchy, had high maysin values that ranged from 0.387% to 0.499%. Hence, maysin production may be regulated by two distinct mechanisms, with one related to the developmental hierarchy and the other based on an independent silk-specific regulation associated with silk scar pigmentation.

Although the first transgenic products largely utilized promoters with constitutive expression patterns, the production of plants for biotechnological uses should ideally target transgene expression to the desired tissues and developmental stages. Endogenous plant promoters of developmentally regulated genes would be expected to provide more precise and controlled expression in specific target tissues. However, our results show that the promoter of the maize *p1* gene, which has been well characterized both genetically and molecularly with respect to its tissue-specific expression (Brink and Styles, 1966; Chopra *et al*., 1996; Sidorenko *et al*., 2000), produced complicated patterns of expression in maize floral organs. The variation in transgene expression patterns amongst *p1* transgenic plants fits a predictable hierarchical ordering. The observation of non-random variation in organ-specific expression patterns amongst *p1* transgenic plants may be indicative of epigenetic plasticity in the *p1* promoter that could account for the high level of phenotypic diversity amongst natural *p1* alleles (Brink and Styles, 1966). Thus, our results demonstrate that the promoters of endogenous plant genes with known tissue specificities may still need to be screened for stable expression patterns prior to widespread application. In addition, we presented a statistical method for determining the significance of transgene expression

patterns, which may be useful for the analysis of other transgene promoters.

In summary, we have analysed maize plants carrying transgenes in which the *P1-rr* or *P1-wr* promoter is used to express the *P1-rr* or *P1-wr* cDNA. The results provide direct evidence that the *p1* gene is competent to induce maysin accumulation in silk tissues. Additionally, the hierarchical patterns of pigmentation observed in transgenic maize plants provide additional support for a model of *p1* regulation in which the timing of a developmentally programmed activation of *p1* expression determines the spectrum of organ pigmentation.

Experimental procedures

Maize materials

The maize *p1* gene described in this paper has previously been designated as *P* or *P1*; here, we use the term *p1* for the locus, *P1* for dominant alleles and *p1* for recessive alleles in accordance with standard maize gene nomenclature (Polacco, 1998). Alleles of the *p1* gene are designated by a two-letter suffix that indicates the presence of phlobaphene pigments in the pericarp and cob: *p1-ww*, white pericarp and white cob; *P1-rr*, red pericarp and red cob; *P1-rw*, red pericarp and white cob; and *P1-wr*, white pericarp and red cob. Allelic designations may also include a third letter in the suffix to indicate the presence or absence of a silk browning phenotype, represented by *b* for browning and *w* for non-browning. In previous experiments, we designed plasmid constructs in which the upstream regulatory regions of *P1-rr* and *P1-wr* were used to express the *P1-rr* and *P1-wr* cDNAs. The two upstream regulatory regions and two cDNA regions were combined in the four possible combinations to generate plasmids designated pRRARR, pRRAWR, pWRARR, pWRAWR and pWRWR, where the first two letters (RR or WR) indicate the source of the upstream regulatory region, and the last two letters indicate the source of the cDNA. The 'A' indicates the presence of the maize *Adh1* intron I in four of the five plasmids (Figure 2; Cocciolone *et al*., 2001). The plasmids were transformed into pre-embryogenic callus derived from immature embryos of the maize HiII line, as described previously (Cocciolone *et al*., 2001). All T_0 transgenic plants were crossed with pollen from the inbred line 4Co63, which carries a *p1-ww* allele, except for the pWRWR transgenic plants, which were crossed with pollen from HiII plants. Tassel glume pigmentation was scored at the time of anthesis, and pericarp, cob and husk pigmentation was scored in mature dried ears. Molecular characterization to confirm independent transformation events and transgene copy numbers has been reported previously (Cocciolone *et al*., 2001).

Maysin analysis

Maize silks were collected 2 days following emergence from the husks. Silks were immediately frozen in liquid nitrogen, stored at –80 °C, lyophilized and analysed for maysin and maysin analogues by reverse-phase HPLC, as described previously (Snook *et al*., 1989).

Statistical analyses

Our primary statistical questions involve the assessment of the significance of the observed associations between silk maysin concentrations and pigmentation patterns or intensities. To properly judge these associations, it is necessary to account for plasmid and event effects in the data. This can be accomplished by examining the significance of covariates of interest when they are added to the following mixed linear model:

$$Y_{ijk} = \mu + \alpha_i + B_{j(i)} + \varepsilon_{ijk} \quad (1)$$

where Y_{ijk} denotes the maysin level of the kth plant from the jth transformation event of the ith plasmid, μ represents the overall mean maysin level for all plants, α_i represents the effect of the ith plasmid, $B_{j(i)}$ represents the effect of the jth event of the ith plasmid ($B_{j(i)}$ is assumed to be independent and normally distributed with a mean of zero and an unknown variance σ_B^2) and ε_{ijk} represents independent random errors that account for all sources of variation not described by the other terms in the model. The use of this mixed model ensures that our statements regarding statistical significance account for: (i) the effects of the four plasmids; (ii) potential correlation amongst the maysin concentrations of plants from any one event; and (iii) the fact that the events observed in this particular experiment are only a sample of the events from a much larger population of potential events. As a result of the imbalance in the number of events per plasmid construct and the number of plants per event, Satterthwaite's (1946) approximation was used to obtain approximate degrees of freedom for all mixed linear model *t*-tests and *F*-tests presented in the 'Results' section.

For each plasmid construct, estimated means and standard errors from the fit of the mixed linear model in Equation (1) are provided at the top of Figure 4. The estimated plasmid means are approximately the mean of the event means within each plasmid. These estimates allow us to make comparisons between plasmids that are undistorted by the varying numbers of plants per event. Comparison of the estimated means shows that plants transformed with the

pWRAWR plasmid have significantly higher mean maysin levels than plants transformed with the pWRARR plasmid (estimated difference = 0.301, $t = 3.07$, d.f. ≈ 13.9, P value = 0.0083). Other differences observed between the plasmid constructs were not statistically significant at the 0.05 level.

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